

Osmoadaptation in Rhizobia: Ectoine-Induced Salt Tolerance

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After having shown that ectoine (a tetrahydropyrimidine) displays osmoprotective properties towards *Escherichia coli* (M. Jebbar, R. Talibart, K. Gloux, T. Bernard, and C. Blanco, J. Bacteriol. 174:5027–5035, 1992), we have investigated the involvement of this molecule in the osmotic adaptation of *Rhizobium meliloti*. Ectoine appeared almost as effective as glycine betaine in improving the growth of *R. meliloti* under adverse osmotic conditions (0.5 M NaCl). Moreover, improvement of growth of rhizobial strains insensitive to glycine betaine was also observed. Ectoine transport proved inducible, periplasmic protein dependent, and, as shown by competition experiments, distinct from the transport of glycine betaine. Medium osmolality had little effect on the uptake characteristics, since the rate of influx increased from 12 to only 20 nmol min⁻¹ mg of protein⁻¹ when NaCl concentrations were raised from 0 to 0.3 or 0.5 M, with a constant of transport of 80 μ M. Natural-abundance ¹³C-nuclear magnetic resonance and radiolabelling assays showed that ectoine, unlike glycine betaine, is not intracellularly accumulated and, as a consequence, does not repress the synthesis of endogenous compatible solutes (glutamate, *N*-acetylglutaminylglutamine amide, and trehalose). Furthermore, the strong rise in glutamate content in cells osmotically stressed in the presence of ectoine suggests that, instead of being involved in osmotic balance restoration, ectoine should play a key role in triggering the synthesis of endogenous osmolytes. Hence, we believe that there are at least two distinct classes of osmoprotectants: those such as glycine betaine or glutamate, which act as genuine osmolytes, and those such as ectoine, which act as chemical mediators.

Rhizobia are soil bacteria which display symbiotic interactions with specific legume hosts. Most of these bacteria are very sensitive to a soil water deficit, which adversely affects their dinitrogen fixation capacity and hence the productivity of the whole legume plant (1, 8, 19, 38). Much attention has been focused during the last decade on the mechanisms of osmotic adaptation in several rhizobium species (18, 41, 42). *Rhizobium meliloti*, the microsymbiont in alfalfa root nodules, has been extensively investigated (5, 6, 22, 36), and the mechanisms governing turgor restoration of bacterial cells growing under hyperosmotic conditions have been partially elucidated. Specifically, concomitant accumulations of potassium and glutamate ions were pointed out as the primary response in *R. meliloti* (6), and the same mechanism has been found in most of the bacterial species investigated thus far (7, 27). Trehalose and the dipeptide *N*-acetylglutaminylglutamine amide (NAGGN) (37) are also accumulated, probably to help the bacterial cells in balancing the high osmotic pressure of the medium. Among a series of exogenously supplied osmoprotectants, glycine betaine and proline betaine proved the most effective (3, 14). Their effect is quite similar to that found in members of the family *Enterobacteriaceae* (23) except that lowering medium osmolality triggers catabolism of betaines in *R. meliloti* (3, 14, 36). A transport system involving a periplasmic glycine betaine-binding protein was identified previously (24, 40). Glycine betaine and proline betaine uptakes are strongly activated during osmotic stress, whereas the enzymes of the catabolic pathways of these molecules are severely repressed (14, 36).

However, it should be mentioned that preculturing the cells in the presence of choline allows for further glycine betaine degradation (36) even in media of high osmolality.

To improve knowledge of osmoregulation of rhizobia, we have investigated the effect of ectoine, which seems to exhibit osmoprotective properties without being accumulated. Extracted and purified from *Brevibacterium linens* (2), an osmotolerant coryneform gram-positive eubacterium, this tetrahydropyrimidine improves the growth of *Escherichia coli* under conditions of high salt concentration (20). Ectoine also osmoprotected several rhizobial species (*Rhizobium leguminosarum*, *Bradyrhizobium japonicum*, and a *Rhizobium* sp.) which proved insensitive to most of the osmoprotectants assayed so far.

MATERIALS AND METHODS

Bacterial strains and culture media. The following rhizobial strains were used in this study: *R. meliloti* 102F34, *B. japonicum* USDA 110, *R. leguminosarum* USDA 2370, *R. leguminosarum* 128C53, *R. leguminosarum* bv. trifolii USDA 2068, and a *Rhizobium* sp. (from *Hedysarum coronarium*). All these strains have previously been used to study the effect of glycine betaine (3). *R. meliloti* RCR 2011 and its derivative GMI 766 ($\Delta nod fixA$), which is defective in glycine betaine, choline, trigonelline, stachydrine, and carnitine catabolism (15), were also investigated. All strains were grown aerobically overnight on MSY (mannitol-salts-yeast extract) rich medium (29) and inoculated at a final concentration of 1% (vol/vol) into minimal medium (LAS) containing 0.1% (wt/vol) sodium aspartate, 0.1% (wt/vol) sodium lactate, and the same salts as in MSY medium. Cells were grown under constant stirring (120 rpm) at 30°C, and the osmolality of the medium was increased by the addition of NaCl when necessary. A solution of 100 mM ectoine extracted and purified from *Brevibacterium linens* (2)

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was filter sterilized through cellulose acetate membranes (0.22- μ m pore size) and added to the culture medium at a final concentration of 1 mM when necessary. Bacterial growth was monitored by A_{420} measurements. Protein contents of the cultures were determined by the method of Lowry et al. (26), with bovine serum albumin used as a standard.

Extraction of cellular solutes. The pellet of freshly harvested and washed cells was extracted at least twice with 80% (vol/vol) ethanol under vigorous magnetic stirring at room temperature for 15 min. After centrifugation, the supernatants (ethanol-soluble fraction) were pooled, filtered through cellulose acetate membranes (0.22- μ m pore size), evaporated to dryness at 40°C, and finally dissolved in distilled water. The ethanol-insoluble pellets contained the intracellular macromolecular components and cell envelopes.

Chromatographic analysis and nuclear magnetic resonance (NMR) spectroscopy. The ethanol-soluble fraction was used directly or after partial purification by passage through a cation-exchange column (10 by 0.5 cm; Bio-Rad AG50 \times 8, H⁺ form). Neutral and acidic compounds were discarded by washing the resin with distilled water, and the cationic fraction (containing amino acids and related molecules) was recovered after elution with 2 M NH₄OH. After concentration, the solutions were analyzed by paper chromatography and thin-layer chromatography, paper high-voltage electrophoresis, and high-pressure liquid chromatography as described by Gouesbet et al. (16). Prior to quantitative analysis, the neutral fraction (containing sugars) was treated as described by Larsen et al. (21) and analyzed for trehalose content. A sample of this fraction was also hydrolyzed in 6 M HCl (110°C for 20 h) or in 9 M KOH, and the resulting amino acids were determined after ninhydrin treatment and monitoring of the A_{570} (17).

The natural-abundance ¹³C-NMR spectra were recorded in the pulsed Fourier transform mode at an operational frequency of 75.4 MHz as previously described (2). Briefly, samples of exponentially growing cells (80 mg of protein) were washed twice with the salt solution of the LAS medium and extracted twice with 80% (vol/vol) ethanol. The solutions were evaporated to dryness, and the residues were dissolved in 0.6 ml of D₂O.

Radiolabelling assays. L-[U-¹⁴C]aspartate (7.4 GBq mmol⁻¹; Commissariat à l'Énergie Atomique, Gif-sur-Yvette, France) was used to label the intracellular solutes accumulated during the growth of *R. meliloti* 102F34 under hyperosmotic conditions. The radioactive amino acid was supplied to cell suspensions at the beginning of the growth in a minimal medium with or without 0.5 M NaCl and with or without 1 mM ectoine. Each sample (10 ml) contained 5.8 mM labelled aspartate (specific radioactivity, 0.2 MBq mmol⁻¹). The suspension was incubated aerobically in test tubes (18 by 1.8 cm) at 30°C. Respired CO₂ was trapped on a strip of filter paper (1 by 3 cm) moistened with 0.2 ml of 5 M KOH. Samples of the suspension were taken at intervals, assayed for their radioactivity (after filtration), and extracted with 80% (vol/vol) ethanol. The radioactivities of the insoluble and soluble fractions were measured separately. An aliquot of the soluble fraction was submitted to two-dimensional paper chromatography as mentioned above. Amino acids in the insoluble pellet were determined after hydrolysis and chromatography as described above. The major labelled compounds from the ethanol-soluble fraction were separated by using an appropriate combination of electrophoresis and chromatography and eluted from the paper. Enzymatic (trehalase) and chemical (acid hydrolysis) treatments were used to confirm the identities of trehalose and the dipeptide.

Chemical synthesis. The dipeptide NAGGN was synthesized by the procedure of Smith and Smith (37) and purified either by passage through a Dowex 50 H⁺ resin or by a

combination of electrophoresis in formic acid (3%, vol/vol) and paper chromatography using *n*-butanol-acetic acid-water (12:3:5, vol/vol) as the solvent mixture. The purity was controlled by ¹³C-NMR spectroscopy and [¹⁴C]glutamate detection in the acid hydrolysate of the dipeptide isolated from [¹⁴C]aspartate-fed cells.

Preparation of labelled osmoprotectants. [¹⁴C]ectoine (10.2 MBq mmol⁻¹) was extracted from cultures of *Brevibacterium linens* grown in minimal medium supplemented with L-[U-¹⁴C]glutamate as described previously (20). [*methyl*-¹⁴C]glycine betaine (2.15 GBq mmol⁻¹) was prepared as described by Perroud and Le Rudulier (30) from [*methyl*-¹⁴C]choline chloride (NEN Research Products, Dupont de Nemours, Les Ulis, France).

Transport assays. Cells grown in minimal medium with or without 1 mM ectoine were centrifuged (5,000 \times g for 10 min), washed twice with an isotonic minimal medium, resuspended to an A_{420} of 5, and maintained at room temperature for 30 min. Ectoine uptake assays were performed as described previously (20), using [¹⁴C]ectoine (10.2 MBq mmol⁻¹) at final concentrations ranging from 10 to 300 μ M in 400 μ l of bacterial suspensions.

Binding assays. Periplasmic proteins were released by the cold-osmotic shock procedure of Neu and Heppel (28) from cells grown in the presence of ectoine. Binding activities were detected by nondenaturing polyacrylamide gel electrophoresis as described by Le Rudulier et al. (24).

Measurement of cell volume. Total cellular volumes were determined by the method of Stock et al. (39), using [*carboxyl*-¹⁴C]inulin (111 MBq g⁻¹) (New England Research Products) and ³H₂O (400 MBq ml⁻¹) (Amersham, Les Ulis, France) as radioactivity markers. Bacterial cells were collected by centrifugation (5,000 \times g for 10 min) and concentrated to an A_{420} of 20. Subsamples (500 μ l) of this suspension were incubated for 30 min with radiolabelled inulin (2 \times 10⁵ dpm) or with tritiated water (2 \times 10⁶ dpm) to determine the extracellular and total volumes of the pellet, respectively. Radioactivity from the supernatant and from the pellet was determined in a liquid scintillation spectrometer.

Nodulation controls. The capacity of the different strains of rhizobia to nodulate *Medicago sativa*, *Pisum sativum*, *Trifolium repens*, and *Glycine max* was checked as previously described (32).

RESULTS

Ectoine-induced salt tolerance in *R. meliloti*. To determine the effect of ectoine on the growth of *R. meliloti* 102F34 under conditions of high osmolarity, the cells were cultivated in minimal LAS medium containing 0.3, 0.5, or 0.7 M NaCl or no NaCl. Ectoine was supplied at a final concentration of 1 mM. An assay using glycine betaine at the same concentration was carried out in parallel for comparison. Data presented in Table 1 show that growth of the bacteria was severely inhibited by 0.5 or 0.7 M NaCl (50 and 95% decrease of growth yield, respectively). Furthermore, the lag phase was longer and the doubling times increased 4- and 15-fold, respectively, in the presence of 0.5 or 0.7 M NaCl. Incorporation of ectoine and glycine betaine into the standard culture medium did not modify growth parameters, whereas a strong improvement in growth was observed in media with high salt concentrations. The effects of glycine betaine and ectoine on cell growth were similar, differing only in a longer lag time observed with ectoine at 0.7 M NaCl. Surprisingly, in the presence of 0.3 M NaCl plus ectoine, the growth yield was 1.5-fold higher than in standard medium. Furthermore, the addition to the LAS

TABLE 1. Effect of ectoine and glycine betaine in improvement of growth of salt-stressed *R. meliloti* 102F34^a

NaCl concn (M)	Result with the indicated osmoprotectant ^b :								
	None			1 mM ectoine			1 mM glycine betaine		
	DT (h)	A _{max}	LT (h)	DT (h)	A _{max}	LT (h)	DT (h)	A _{max}	LT (h)
0.0	4.3	4	0.5	4.5	4.9	0.5	4.5	4.4	0.5
0.3	6.3	4	4	5.3	6	3	5	4.5	2
0.5	18	2.1	5.5	7.5	4.1	6	6.5	4.1	3
0.7	60	0.2	60	18	0.8	22	14	0.9	10

^a Cells were grown aerobically in LAS minimal medium containing lactate and aspartate as C and N sources.

^b DT, doubling time; A_{max}, maximal absorbance; LT, growth lag time. Data are the means of triplicate assays (standard deviations never exceeded 5%).

medium of 1 mM glutamate, α-ketoglutarate, or succinate failed to mimic the ectoine salt tolerance effect. Hence, ectoine appeared almost as effective as glycine betaine in improvement of growth of salt-stressed *R. meliloti* cells.

Characteristics of ectoine uptake in *R. meliloti*. Cells grown in media without ectoine did not accumulate significant amounts of label from [¹⁴C]ectoine until 40 min after ectoine was added, regardless of osmolarity. Beyond this delay, the uptake rate (5.8 nmol min⁻¹ mg of protein⁻¹) was quite similar to that observed for cells grown beforehand in the presence of 1 mM ectoine (7.6 nmol min⁻¹ mg of protein⁻¹) (Fig. 1). The inducibility of a transport system by ectoine was therefore investigated. As expected, chloramphenicol-treated cells did not show any significant uptake activity (0.06 nmol min⁻¹ mg of protein⁻¹). The induction seemed specific for ectoine since glycine betaine neither triggered nor repressed ectoine uptake.

Kinetic constants indicate that ectoine uptake activity was increased little by raising medium osmolarity. Indeed, the

maximal rate of ectoine uptake in cells grown in LAS medium without NaCl was 12 nmol min⁻¹ mg of protein⁻¹, and that in the presence of 0.3 or 0.5 M NaCl was 20 nmol min⁻¹ mg of protein⁻¹. In contrast, the K_m remained constant (80 μM) irrespective of NaCl concentration. To assess whether the ectoine transport system is distinct from that of glycine betaine, we examined the effect of unlabelled competitors on [¹⁴C]ectoine and [¹⁴C]glycine betaine uptakes. No competitive effects were observed when glycine betaine, choline, or proline was used in 10- to 100-fold excess with respect to [¹⁴C]ectoine. Under these conditions, only unlabelled ectoine was capable of reducing [¹⁴C]ectoine uptake by 90 and 100%, respectively. Similarly, [¹⁴C]glycine betaine uptake was affected only by the presence of an excess of unlabelled glycine betaine. Thus, the ectoine uptake system should be considered distinct from that of glycine betaine.

Since *R. meliloti*, like other gram-negative eubacteria, is capable of transporting a variety of organic solutes, including glycine betaine (24, 40), through periplasmic protein-dependent transport systems, the periplasmic shock fluid from *R. meliloti* cells was analyzed by electrophoresis and labelling with [¹⁴C]ectoine or [¹⁴C]glycine betaine for the detection of the corresponding binding proteins. Figure 2 shows that two distinct protein bands were labelled with ectoine and glycine betaine, indicating that the periplasm of *R. meliloti* contains an ectoine-binding protein distinct from the glycine betaine-binding protein.

The fate of ectoine. To specify the fate of ectoine, we first analyzed the ability of *R. meliloti* 102F34 to utilize ectoine as a nitrogen or carbon source in LAS medium deprived of lactate and aspartate but containing various ectoine concentrations (5 μM to 5 mM). It appeared that ectoine was used as the sole

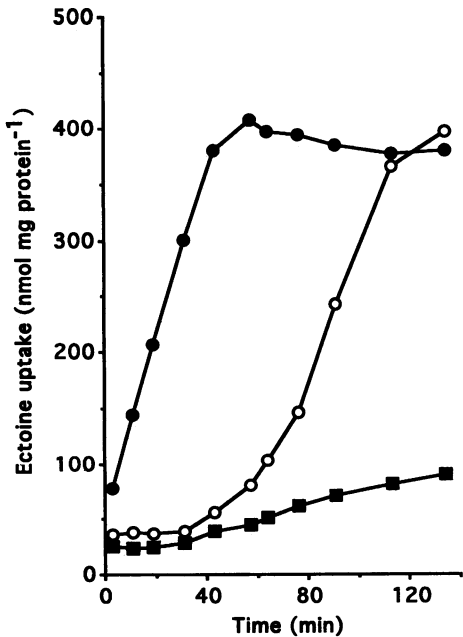


FIG. 1. Induction of ectoine transport in *R. meliloti* cells. [¹⁴C]ectoine (3.95 MBq mmol⁻¹; 290 μM) uptake assays were carried out in LAS medium. Prior to each assay, the cells were grown in LAS medium to an A₄₂₀ of 0.6 and then transferred to basal LAS medium with (●) or without (○) 1 mM ectoine or to basal LAS medium containing 1 mM ectoine and 100 μg of chloramphenicol ml⁻¹ (■) and incubated for 4 h.

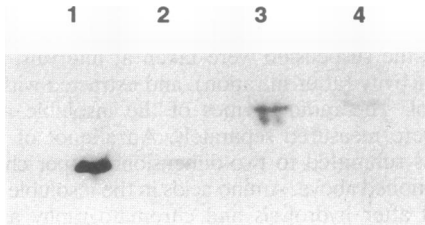


FIG. 2. Identification by nondenaturing polyacrylamide gel electrophoresis of an ectoine-binding protein in the periplasmic fluid of *R. meliloti*. Samples (35 μg) of periplasmic proteins from ectoine-induced cells were incubated with [¹⁴C]glycine betaine or [¹⁴C]ectoine as described in Materials and Methods. Lanes 1 and 2, proteins incubated with 7 μM [¹⁴C]glycine betaine (2.15 and 0.01 GBq mmol⁻¹, respectively); lanes 3 and 4, proteins incubated with 10 μM [¹⁴C]ectoine (0.115 and 0.01 GBq mmol⁻¹, respectively).

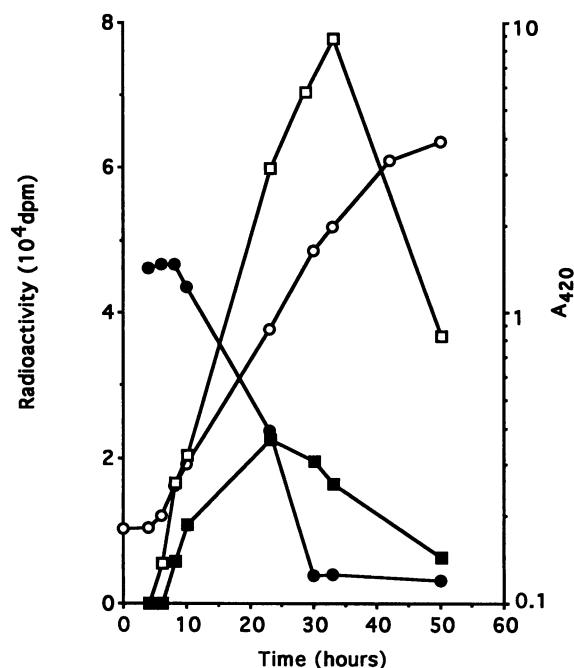


FIG. 3. The fate of [^{14}C]ectoine in *R. meliloti* 102F34. Cells were grown in 0.5 M NaCl-LAS medium supplemented with 1 mM [^{14}C]ectoine ($0.83 \text{ MBq mmol}^{-1}$), and radioactivity was determined in the different fractions during the growth. Symbols: \circ , A_{420} ; \bullet , radioactivity in the medium (in disintegrations per minute per milliliter); \square , radioactivity of the ethanol-insoluble material (in disintegrations per minute per milligram of protein); \blacksquare , radioactivity of the ethanol extract (in disintegrations per minute per milligram of protein).

nitrogen and carbon source but gave rise to significant growth only at concentrations above 0.1 mM. Under these conditions, growth yield was proportional to ectoine concentration. When cells were grown in minimal medium containing ectoine as both carbon and nitrogen sources in the presence of 0.3 and 0.5 M NaCl, growth yield and growth rate were both affected by the medium osmolality. However, *R. meliloti* GMI 766, which is defective in the catabolism of various osmoprotectants, including glycine betaine, carnitine, choline, and stachydrine (15), was still capable of growing on ectoine as the carbon and nitrogen source. Thus, the assimilation pathway of ectoine is distinct from that described for the compounds mentioned above.

To examine the intracellular fate of ectoine, bacterial cells were grown to an A_{420} of 0.6 in minimal medium containing 0.5 M NaCl and 1 mM [^{14}C]ectoine and the radioactivity of cellular ethanol extracts, insoluble material, and evolved CO_2 was determined. Surprisingly, radioactivity was detected mainly in the insoluble (32%) and in the CO_2 (56%) fractions, whereas only 12% was found in the soluble fraction. Similar data were obtained with cells grown in the presence of 0.3 or 0.7 M NaCl. Time course experiments were then performed with cells grown in 0.5 M NaCl to analyze more thoroughly the phenomenon. Figure 3 clearly shows that the incorporation of the radiocarbon into the insoluble material starts at the earliest stages of growth and increases until the medium is depleted of ectoine. Furthermore, in the ethanol-insoluble fraction, the radioactivity was recovered within several amino acids but ectoine was not detected. Radioactivity in the ethanol extract never exceeded a third of that in insoluble material. Chromatographic analysis of the ethanol extract showed that ectoine

presented only traces of radioactivity and reached a maximal intracellular concentration of 12 mM. These data indicate that ectoine is catabolized during cell growth even under conditions of high osmolality and that its carbons are partially incorporated into cell macromolecules. It is noteworthy that the maximal intracellular ectoine concentration which was recorded (12 mM) cannot counterbalance the osmotic pressure developed by the presence of 0.5 M NaCl in the external medium.

Identification of the major intracellular solutes. Since ectoine, though displaying osmoprotective properties, is not accumulated by *R. meliloti*, a natural-abundance ^{13}C -NMR analysis was undertaken to identify the main intracellular solutes in bacterial cells grown in the presence or absence of 0.5 M NaCl and/or 1 mM ectoine. Except for a small peak corresponding to trehalose, the spectrum of ethanolic extracts of cells grown in the absence of NaCl (Fig. 4) displayed no major signal while that of cells grown with 0.5 M NaCl exhibited several peaks. The calculated chemical shifts at 184.05, 177.31, 57.36, 36.15, and 29.67 ppm (with tetramethylsilane as a standard) were similar to those of commercial glutamate. Resonances at 95.93, 75.25, 74.88, 73.77, 72.41, and 63.26 ppm correspond to the glucose moiety of trehalose. Furthermore, a series of signals at 180.60, 180.54, 178.50, 177.21, 176.47, 55.99, 55.60, 33.77, 29.34, and 24.39 ppm might correspond to the dipeptide NAGGN (see reference 37). To confirm this hypothesis, the spectra of NAGGN purified from *R. meliloti* and of NAGGN obtained by chemical synthesis were compared. Since the two spectra proved similar, NAGGN is, with glutamate and trehalose, the main accumulated solute in *R. meliloti* cells in the presence of 0.5 M NaCl. It should be noted, however, that ectoine, contrary to the other major solutes mentioned above, was not revealed by NMR spectroscopy in cells of *R. meliloti* grown in the presence of 1 mM ectoine in a medium containing 0.5 M NaCl. Moreover, it did not repress the accumulation of the major endogenous solutes.

Determination of intracellular glutamate, NAGGN, and trehalose. Since only three compounds were revealed by NMR spectroscopy, they probably are the only organic solutes involved in cell turgor maintenance. The levels of intracellular glutamate, NAGGN, and trehalose were determined for ethanolic extracts of cells grown in LAS medium with or without 0.5 M NaCl and/or 1 mM ectoine. The variations of the intracellular levels of the different compounds during bacterial growth were estimated.

When cells were grown in a medium of low osmolality, the levels of glutamate, NAGGN, and trehalose were quite constant during the exponential growth phase, with 80, 50, and 40 nmol mg of protein $^{-1}$, respectively. When cells were grown in the presence of 0.5 M NaCl (Fig. 5), the trehalose content rapidly reached 200 nmol mg of protein $^{-1}$ and then increased slowly up to 250 nmol mg of protein $^{-1}$. Glutamate was the only accumulated amino acid, with levels of 175 nmol mg of protein $^{-1}$ at the beginning of growth and 520 nmol mg of protein $^{-1}$ in the middle of the exponential growth phase and then a slow decrease down to 260 nmol mg of protein $^{-1}$ at the end of the growth. The NAGGN level increased during bacterial growth, up to 400 nmol mg of protein $^{-1}$ at the early stationary growth phase. When cells were grown in the presence of 0.5 M NaCl and 1 mM ectoine, a strong increase of the intracellular glutamate level was observed. Indeed, the glutamate content reached 950 nmol mg of protein $^{-1}$ at the end of the exponential growth phase and decreased sharply during the stationary phase. The trehalose content steadily increased during the exponential growth phase from 50 to 300 nmol mg of protein $^{-1}$. The data regarding NAGGN were similar to

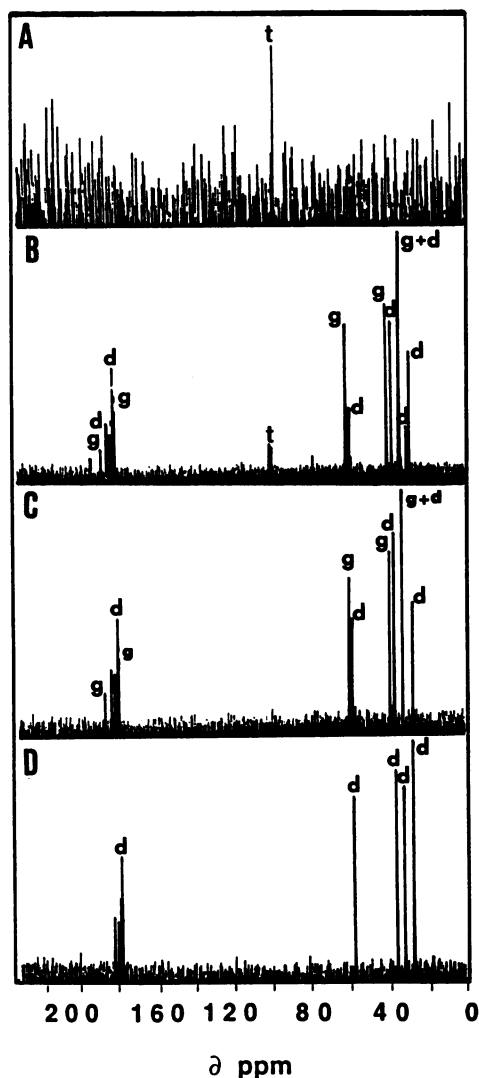


FIG. 4. Natural-abundance ^{13}C -NMR spectra of ethanolic extracts from *R. meliloti* cells. Ethanolic extracts were prepared and analyzed as described in Materials and Methods with bacterial cells grown in LAS medium (A) or in 0.5 M NaCl-LAS medium without (B) or with (C) 1 mM ectoine. The spectrum of synthetic NAGGN is also given (D). d, dipeptide (NAGGN); g, glutamate; t, trehalose. Each spectrum was obtained under identical conditions (amount of cells corresponding to 80 mg of protein; 1,024 scans). Only the signals from panel A were amplified (about fourfold).

those obtained with cells grown in the absence of ectoine. Indeed, the dipeptide reached the same maximal intracellular level ($400 \text{ nmol mg of protein}^{-1}$) in the middle of the exponential phase and then remained quite constant during the growth.

To take into consideration the actual intracellular concentration, the total cell volume was determined at the early, mid-, and late exponential phases of growth. For cells grown on no-salt medium with or without ectoine, the total cell volume was $5 \pm 0.4 \mu\text{l mg of protein}^{-1}$ (mean \pm standard deviation); at 0.5 M NaCl without ectoine, no significant change was observed during growth ($2.5 \pm 0.2 \mu\text{l mg of protein}^{-1}$), whereas in the presence of ectoine the cell volume increased steadily from 2.5 ± 0.2 to $3.8 \pm 0.3 \mu\text{l mg of protein}^{-1}$ at the

beginning and at the end of growth, respectively. Consequently, the calculated concentration of intracellular glutamate was quite constant at about 250 mM. It should be mentioned that this value is somewhat underestimated since the cytoplasmic volume could represent only 80% of the total cell volume (39).

To confirm these observations, cells were grown in the presence of [^{14}C]aspartate in 0.5 M NaCl-LAS medium with or without ectoine. [^{14}C]NAGGN represented 50% of the radioactivity in the earliest stages of growth, and its level decreased slightly with time. In contrast, the percentage of [^{14}C]trehalose increased during the growth, more slowly in the presence than in the absence of ectoine, while [^{14}C]glutamate never accounted for more than 20% of the radioactivity. These data indicate that aspartate is the main source of carbons for NAGGN and trehalose, whereas aspartate carbons contribute poorly to glutamate synthesis.

Effect of ectoine on other rhizobium strains. The effect of ectoine on *R. meliloti* 102F34 revealed in this study is different from that observed with other osmoprotectants. To determine whether this phenomenon is general or strain specific, we examined the osmoprotective activity of ectoine on several other rhizobial strains. Seven strains of *Rhizobium* and one strain of *Bradyrhizobium* were therefore investigated for their potential salt tolerance in the absence or presence of 1 mM ectoine (Table 2). All these strains were salt sensitive and showed a two- to fivefold increase of the generation time in the presence of high salt concentrations. Addition of ectoine improved the growth in high-salt medium and reduced by two- to threefold the doubling time of all the strains except for one *Rhizobium* sp. (from *H. coronarium*). Further experiments showed that this strain was unable to take up [^{14}C]ectoine. The incorporation of 1 mM ectoine into culture media deprived of NaCl did not significantly modify the growth rate. Moreover, as for *R. meliloti*, supplying the different rhizobial cells with [^{14}C]ectoine resulted in uptake without a significant intracellular accumulation of this solute.

Controlling for the nodulating capacity of these different strains by using *M. sativa*, *P. sativum*, *T. repens*, and *G. max* as host plants showed that *R. meliloti* induced nodule formation only on *Medicago* roots, whereas the other rhizobia nodulated only their corresponding symbiotic plant partners.

DISCUSSION

In this report, we provide evidence that *R. meliloti* 102F34 is protected by ectoine against hyperosmotic stress, as previously shown with glycine betaine (3). However, unlike glycine betaine, ectoine was also capable of protecting rhizobia insensitive to most of the osmoprotectants assayed so far. Only exogenously supplied trehalose has been found to more or less protect a few strains of *Rhizobium fredii* (at 0.34 M NaCl) and *B. japonicum* (at only 0.08 M NaCl) (11). The effect of glycine betaine does not seem general, since a slight improvement in the growth of *B. japonicum* RCR 3407 in the presence of 0.08 M NaCl has already been reported (10) whereas no beneficial effect was found for other *Bradyrhizobium* strains (reference 3 and this study).

It is noteworthy that among the solutes which alleviate osmotic growth inhibition, ectoine seems to play a peculiar role. While there is a generally good correlation between intracellular accumulation of the supplied osmoprotectant and growth restoration under adverse conditions (7), our data indicate that this does not hold true for ectoine in the case of *R. meliloti*. It has been reported that this molecule is accumulated in *Ectothiorhodospira halochloris* (13) and in a variety of

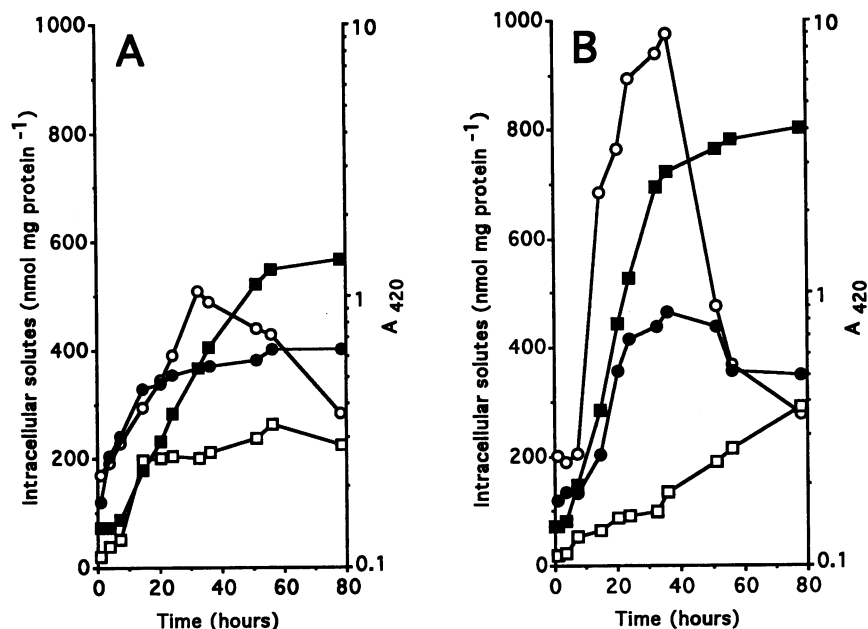


FIG. 5. Determination of the concentrations of the major intracellular solutes during the growth of *R. meliloti*. Cells were grown in 0.5 M NaCl-LAS medium (A) or in 0.5 M NaCl-LAS medium containing 1 mM ectoine (B). Glutamate, NAGGN, and trehalose were determined as described in Materials and Methods. Symbols: ■, A₄₂₀; ○, glutamate; ●, NAGGN; □, trehalose.

halotolerant bacteria (20, 35) during growth under high salinity. Recently, we identified ectoine in *Brevibacterium linens* and showed that this molecule was by far the most prominent solute synthesized and accumulated by this coryneform gram-positive eubacterium as a function of medium osmolality (2). Furthermore, we have demonstrated that exogenously supplied ectoine was effective in relieving osmotic stress in *E. coli* (20).

In *R. meliloti*, the ectoine transport system appears specific, inducible, and periplasmic protein dependent and, similar to glycine betaine, ectoine can also be catabolized. The use of strain GMI 766, which has the genes essential for the catabolism of other known osmoprotectants such as trigonelline, stachydrine, and carnitine deleted (15), led to the demonstration that ectoine catabolism may follow a different pathway.

TABLE 2. Improvement of growth of *Rhizobia* and *Bradyrhizobia* strains by ectoine under high-osmolality conditions^a

Rhizobium	Strain	Doubling time (h) in:		
		Minimal medium	Minimal medium + NaCl ^b	
			Without ectoine	With ectoine ^c
<i>R. meliloti</i>	102F34	4	20 (0.5)	8
	2011	5	35 (0.5)	16
<i>B. japonicum</i>	110	10	35 (0.2)	10
<i>R. leguminosarum</i> Biovar viciae	2370	15	40 (0.2)	16
	128C53	12	30 (0.2)	14
Biovar trifolii	2068	14	35 (0.2)	18
<i>Rhizobium</i> sp. (<i>H. coronarium</i>)		8	18 (0.2)	18

^a Bacteria were grown as described in Materials and Methods. Data are the means of triplicate assays (standard deviations never exceeded 5%).

^b Final NaCl concentrations (molar) are indicated in parentheses.

^c Ectoine was used at a final concentration of 1 mM.

Furthermore, no ectoine-derived metabolite was ever found accumulated.

Under high-osmolality conditions (0.5 M NaCl), *R. meliloti* did not accumulate ectoine at concentrations higher than 12 mM, which is obviously too low to make any significant contribution to cell osmotic equilibrium. Intracellular-solute analysis of salt-stressed cells in a medium containing or lacking ectoine showed that this molecule, unlike the majority of exogenously supplied osmoprotectants, did not suppress the accumulation of endogenous solutes at all. Thus, glutamate, trehalose, and the dipeptide NAGGN were still present at high levels even in the presence of ectoine. Moreover, the intracellular glutamate level strongly increased during the growth of the cells, while the trehalose level increased towards the end of the exponential growth phase.

Interestingly, the knowledge of cell volume allowed demonstration of the parallel between glutamate content and cell volume increases in cells grown in the presence of ectoine; moreover, glutamate by itself appeared to contribute to balance by approximately 50% of the osmotic pressure of the external medium (0.5 M NaCl). Therefore, its participation in restoring cell turgor of stressed cells could not be neglected. Glutamate, trehalose, and NAGGN have already been shown to contribute together to the osmotic equilibrium of stressed cells of both *R. meliloti* (37) and *Pseudomonas aeruginosa* (9). In the latter species, glutamate remains the most abundant intracellular osmolyte and exogenously supplied glycine betaine does not totally obliterate (even at 0.7 M NaCl) the accumulation of endogenous osmolytes as it does in *E. coli* stressed cells (21).

Due to its high relative abundance and to the fluctuations of its intracellular amounts in response to osmotic constraints, glutamate seems to play a pivotal function in cell osmoregulation. Its paramount importance in the primary response to hyperosmotic conditions is universally accepted (for reviews, see references 4 and 7), and its role in gene transcription,

together with K^+ ions, has been shown in vitro (33). In *R. meliloti* 102F34 cultivated in the presence of 0.4 M NaCl, the intracellular glutamate content increased more than eightfold compared with that of the control without added salt. The increase in glutamate level was accompanied by a concomitant increase in potassium content, with neither glutamate dehydrogenase nor glutamate synthase (the enzyme working with glutamine synthetase in the alternative ammonia assimilating pathway) being responsible for the enhancement of glutamate synthesis (6). Only the specific inhibition of aminotransferases succeeded in blocking the synthesis and accumulation of glutamate in stressed *R. meliloti* cells. Therefore, it should prove interesting to investigate further the role of these enzymes in osmoregulation phenomena in *R. meliloti*.

Since ectoine exhibits osmoprotection in *R. meliloti* without being accumulated by the cells, it is tempting to speculate that its mechanism of action is situated at the gene expression level. In this hypothesis, ectoine would trigger, after a lag period, the expression of a set of *osm* genes (i.e., those encoding the enzymes involved in endogenous osmolyte biosyntheses). This hypothesis is consistent with the fact that several genes have their transcription turned on by signals from the cell environment. Specifically, *nod* genes in *R. meliloti* have been shown to respond positively, in addition to a variety of phenolic compounds, to trigonelline and to stachydrine, two betaines occurring in the alfalfa host plant and released in their rhizosphere (31, 34). Further investigations are needed to elucidate the ectoine osmoprotection mechanism.

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REFERENCES

1. Aparicio-Tejo, P., and M. Sánchez-Díaz. 1982. Nodule and leaf nitrate reductases and nitrogen fixation in *Medicago sativa* L. under water stress. *Plant Physiol.* **69**:479–482.
2. Bernard, T., M. Jebbar, Y. Rassouli, S. Himdi-Kabbab, J. Hamelin, and C. Blanco. 1993. Ectoine accumulation and osmotic regulation in *Brevibacterium linens*. *J. Gen. Microbiol.* **139**:129–138.
3. Bernard, T., J.-A. Pocard, B. Perroud, and D. Le Rudulier. 1986. Variations in the response of salt-stressed *Rhizobium* strains to betaines. *Arch. Microbiol.* **143**:359–364.
4. Booth, I. R. 1993. Regulation of gene expression during osmoregulation: the role of potassium glutamate as a secondary signal of osmotic stress, p. 309–331. In E. P. Bakker (ed.), *Alkali cation transport systems in prokaryotes*. CRC Press, Inc., Boca Raton, Fla.
5. Botsford, J. L. 1984. Osmoregulation in *Rhizobium meliloti*: inhibition of growth by salts. *Arch. Microbiol.* **137**:124–127.
6. Botsford, J. L., and T. A. Lewis. 1990. Osmoregulation in *Rhizobium meliloti*: production of glutamic acid in response to osmotic stress. *Appl. Environ. Microbiol.* **56**:488–494.
7. Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* **53**:121–147.
8. Dejong, T. M., and D. A. Phillips. 1982. Water stress effects on nitrogen assimilation and growth of *Trifolium subterraneum*. *Plant Physiol.* **69**:416–420.
9. D'Souza-Ault, M. R., L. T. Smith, and G. M. Smith. 1993. Roles of *N*-acetylglutamylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress. *Appl. Environ. Microbiol.* **59**:473–478.
10. Elsheikh, E. A. E., and M. Wood. 1989. Response of chickpea and soybean rhizobia to salt: osmotic and specific ion effects of salts. *Soil Biol. Biochem.* **21**:889–895.
11. Elsheikh, E. A. E., and M. Wood. 1990. Rhizobia and bradyrhizobia under salt stress: possible role of trehalose in osmoregulation. *Lett. Appl. Microbiol.* **10**:127–129.
12. Galinski, E. A. 1993. Compatible solutes of halophilic eubacteria: molecular principles, water-solute interaction, stress protection. *Experientia* **49**:487–496.
13. Galinski, E. A., H. P. Pfeiffer, and H. G. Trüper. 1985. 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid: a novel cyclic amino acid from halophilic phototrophic bacterium *Ectothiorhodospira*. *Eur. J. Biochem.* **149**:135–139.
14. Gloux, K., and D. Le Rudulier. 1989. Transport and catabolism of proline betaine in salt-stressed *Rhizobium meliloti*. *Arch. Microbiol.* **151**:143–148.
15. Goldmann, A., C. Boivin, V. Fleury, B. Message, L. Lecœur, M. Maille, and D. Tepfer. 1991. Betaine use by rhizosphere bacteria: genes essential for trigonelline, stachydrine, and carnitine catabolism in *Rhizobium meliloti* are located on Psym in the symbiotic region. *Mol. Plant Microbe Interact.* **4**:571–578.
16. Gouesbet, G., C. Blanco, J. Hamelin, and T. Bernard. 1992. Osmotic adjustment in *Brevibacterium ammoniagenes*: pipecolic acid accumulation at elevated osmolalities. *J. Gen. Microbiol.* **138**:959–965.
17. Hirs, C. H. W. 1967. Detection of peptides by chemical methods. *Methods Enzymol.* **11**:325–329.
18. Hua, S.-S. T., V. Y. Tsai, G. M. Lichens, and A. T. Noma. 1982. Accumulation of amino acids in *Rhizobium* sp. strain WR1001 in response to sodium chloride salinity. *Appl. Environ. Microbiol.* **44**:135–140.
19. Huang, C. Y., J. S. Boyer, and L. N. Vanderhoes. 1975. Acetylene reduction (nitrogen fixation) and metabolic activity of soybean having various leaf and nodule water potential. *Plant. Physiol.* **56**:222–227.
20. Jebbar, M., R. Talibart, K. Gloux, T. Bernard, and C. Blanco. 1992. Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristics. *J. Bacteriol.* **174**:5027–5035.
21. Larsen, P. I., L. K. Sydnæs, B. Landfald, and A. R. Strøm. 1987. Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. *Arch. Microbiol.* **147**:1–7.
22. Le Rudulier, D., and T. Bernard. 1986. Salt tolerance in *Rhizobium*: a possible role for betaines. *FEMS Microbiol. Rev.* **39**:67–72.
23. Le Rudulier, D., and L. Bouillard. 1983. Glycine betaine, an osmotic effector in *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae*. *Appl. Environ. Microbiol.* **46**:152–159.
24. Le Rudulier, D., K. Gloux, and N. Riou. 1991. Identification of an osmotically induced periplasmic glycine betaine-binding protein from *Rhizobium meliloti*. *Biochim. Biophys. Acta* **1061**:197–205.
25. Lippert, K., and E. A. Galinski. 1992. Enzyme stabilization by ectoine type compatible solutes: protection against heating, freezing and drying. *Appl. Microbiol. Biotechnol.* **37**:61–65.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
27. Measures, J. C. 1975. Role of amino acids in osmoregulation of nonhalophilic bacteria. *Nature (London)* **257**:398–400.
28. Neu, H., and L. A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J. Biol. Chem.* **240**:3685–3692.
29. O'Gara, F., and K. T. Shanmugam. 1976. Regulation of nitrogen fixation by rhizobia: export of fixed N_2 as NH_4^+ . *Biochim. Biophys. Acta* **437**:313–321.
30. Perroud, B., and D. Le Rudulier. 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. *J. Bacteriol.* **161**:393–401.
31. Phillips, D. A., C. M. Joseph, and C. A. Maxwell. 1992. Trigonelline and stachydrine released from alfalfa seeds activate NodD2 protein in *Rhizobium meliloti*. *Plant Physiol.* **99**:1526–1531.
32. Pocard, J.-A., T. Bernard, G. Goas, and D. Le Rudulier. 1984. Restauration partielle, par la glycine bétaine et la proline bétaine, de l'activité fixatrice d'azote de jeunes plantes de *Medicago sativa* L., soumises à un stress hydrique. *C. R. Acad. Sci. Paris* **298**:477–480.
33. Ramirez, R. M., W. S. Prince, E. Bremer, and M. Villarejo. 1989. *In vitro* reconstitution of osmoregulated expression of *proU* of

- Escherichia coli*. Proc. Natl. Acad. Sci. USA **86**:1153–1157.
34. **Schmidt, J., M. John, U. Wieneke, H. D. Krussmann, and J. Shell.** 1986. Expression of the nodulation gene *nodA* in *Rhizobium meliloti* and localization of the gene product in the cytosol. Proc. Natl. Acad. Sci. USA **83**:9581–9585.
 35. **Severin, J., A. Wohlfarth, and E. A. Galinski.** 1992. The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. J. Gen. Microbiol. **138**: 1629–1638.
 36. **Smith, L. T., J.-A. Pocard, T. Bernard, and D. Le Rudulier.** 1988. Osmotic control of betaine biosynthesis and degradation in *Rhizobium meliloti*. J. Bacteriol. **170**:3142–3149.
 37. **Smith, L. T., and G. M. Smith.** 1989. An osmoregulated dipeptide in stressed *Rhizobium meliloti*. J. Bacteriol. **171**:4714–4717.
 38. **Sprent, J. I.** 1972. The effects of water stress on nitrogen-fixing root nodules. IV. Effects on whole plants of *Vicia faba* and *Glycine max*. New Phytol. **71**:603–611.
 39. **Stock, J. B., B. Rauch, and S. Roseman.** 1977. Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. **252**:7850–7851.
 40. **Talibart, R., M. Le Hénaff, T. Bernard, and H. Wróblewski.** 1990. Identification of bacterial periplasmic glycine betaine-binding protein after electrophoresis and affinity labeling. J. Biochem. Biophys. Methods **21**:155–164.
 41. **Yap, S. F., and S. T. Lim.** 1983. Response of *Rhizobium* sp. UMKL 20 to sodium chloride stress. Arch. Microbiol. **135**:224–228.
 42. **Yelton, M. M., J. T. Mulligan, and S. R. Long.** 1987. Expression of *Rhizobium meliloti nod* genes in *Rhizobium* and *Agrobacterium* backgrounds. J. Bacteriol. **169**:3094–3098.